Ann Ig 2022 Mar-Apr; 34(2): 177-183. doi: 10.7416/ai.2022.2477

Isolation and identification of *Legionella* spp. from non-hospital facilities: a preliminary one-year surveillance study in the urban area of Pesaro-Urbino (Central Italy)

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Key words: Legionella spp., isolation, survey study, Central Italy
Parole chiave: Legionella spp., isolamento, sorveglianza, Italia Centrale

Abstract

Background. *Legionella is considered one of the most important causes of potentially preventable morbidity and mortality. These microorganisms are ubiquitous, but incomplete information is available on the geographic distribution of Legionella species in our region.*
Study design. *For the mentioned reasons, in this work the distribution of Legionella spp. in non-hospital facilities of the urban area of Pesaro-Urbino (Central Italy), including public fountains, residential buildings, public and private offices and retirement homes, was investigated.*
Methods. *A total of 298 water samples were collected from the different facilities and subjected to standard Legionella isolation and identification protocols.*
Results. *As reported, 17.8% of the collected water samples resulted positive for Legionella spp. (28.6% from retirement homes, 21.3% from residential buildings, 15.3% from private and public offices). The highest percentage of positive samples (14.4%) was found in hot water from retirements homes (58.8%) and residential buildings (31.8%); the most frequent isolated serogroups were L. pneumophila 2-14 (71.7%).*
Conclusions. *This work is the first describing the distribution of Legionella spp. in non-hospital facilities in the province of Pesaro-Urbino, and highlights a condition of potential risk for susceptible categories. From our data, we can point that a regular and constant control to prevent microbiological risk from legionellosis, particularly in facilities housing the elderly, is recommended.*

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Annali di Igiene : Medicina Preventiva e di Comunità (Ann Ig)
ISSN 1120-9135 https://www.annali-igiene.it

Introduction

Legionella is a Gram-negative bacterium widely present in soil and freshwater environments, that can also contaminate man-made aquatic water systems. In these environments, many factors, such as aged plumbing, low flow rate, water stagnation, storage tanks or surface materials, roughness, physicochemical features, can influence the growth of *Legionella* (1-4). Typically, this microorganism can replicate between 25°C and 42°C but, surviving also at temperatures up to a maximum of 57°-63°C (5), *Legionella* can be responsible for both nosocomial and community-acquired infections (6, 7). This microorganism causes different distinct clinical diseases: Pontiac fever, self-limited flu-like illness with no associated mortality, the severe manifestation of Legionnaires' Disease (LD) including symptoms of pneumonia associated with mortality, and sub-clinical and silent infection (8-10). The main risk factors for acquiring *Legionella* infections are chronic diseases, diabetes, various conditions associated with immunodeficiency, but also increasing age and male sex (10). All the mentioned infections are acquired by inhalation, aspiration, or micro-aspiration of *Legionella*-carrying aerosols originated by water spraying or by gurgling air through contaminated water (11) as well as by showerheads, certain medical equipment (e.g. respiratory equipment), cooling towers, hydrotherapy equipment, decorative fountains (7). Outbreaks of waterborne disease affect a large number of persons and drinking water can contribute to background rates of disease in non-outbreak situations (4). Indeed, outbreaks responsible for a small portion of the total cases and sporadic cases are much more common (12).

The legionellosis'surveillance in Europe is under the authority of the European Centre for Disease Prevention and Control (ECDC) and, in Italy, of the Italian National Institute of Health (ISS). The latest data related to 2017, reported 9,238 cases of Legionaries' disease (notification rate 1.6 per 100,000 inhabitants), of whom 2.013 cases occurred in Italy (notification rate 3.2 per 100,000 inhabitants). As in the previous year, 71% were community-acquired, 20% were travel-associated, 7% related to healthcare facilities and 2% to other settings. It can be noted that there was a significant general increment of cases compared to 2016, when 7,069 cases of LD (notification rate of 1.4 per 100,000 inhabitants) were registered in EU/EEA (1,680 cases occurred in Italy, with a notification rate 2.8 per 100,000 inhabitants) (13). In this context, unfortunately, Italy is one of the countries with the largest number of cases, from 1,535 in 2015 to 2,013 cases in 2017, and an average annual incidence of 2.8 cases per 100,000 inhabitants. These data stress the relevance of *Legionella* infections in our country and the need for continuous control, as well as for the biological environmental risk assessment. Already in 2000, the ISS issued its first guidelines for the prevention and control of legionellosis and, in 2005, these guidelines were followed by specific instructions for the laboratories involved in the microbiological diagnosis and environmental control, and also for tourist accommodations, and spas. Finally, in 2015, all the national recommendations, including those for hospitals, were incorporated, by the Italian Ministry of Health, into a unique updated document for legionellosis control in all the settings (14), with indications for prevention and control of *Legionella* in the different contexts and how to operate in the case of positive samples.

In this direction, this study aimed to assess the presence of *Legionella* spp. in water systems networks in the urban area of Pesaro-Urbino (Central Italy) in a preliminary one-year-surveillance study (January-December 2018). For this, water samples collected from public fountains, residential buildings, public and private

offices and retirement homes, were subjected to *Legionella* isolation and identification protocols to obtain more information on *Legionella* colonization and distribution in these facilities.

Methods

Samples collection

Following the requests of some local administrators of controlling water quality, a one-year survey was performed from January 2018 to December 2018 in the urban area of Pesaro-Urbino (Central Italy). A total of 298 water samples were collected from public fountains (6.1%), residential buildings (25.2%), public and private offices (54.7%) and retirement homes (14.1%). The supplied water is the water distributed by municipal network and is normally used by the facilities for all normal domestic and human uses.

Sampling method

Hot and cold water samples were collected in agreement with the Italian Guidelines for prevention and control of legionellosis (14). All water samples were taken without flaming the outlet point and without previously running the water ("instantaneous sample"), to simulate the theoretical user's exposure (15). One L samples were collected in sterile polyethylene bottles supplemented with 100 mg/L sodium thiosulphate to neutralize the chlorine (15, 16). In the case of public fountains only cold water was considered. All the samples were transported to the laboratory, protected from light and at room temperature; the microbiological analysis was performed within 24 h.

Microbiological analysis

Each water sample was filtered through 0.2 μm pore-sized cellulose nitrate filter (Sartorius Stedim Biotech, Göttingen, Germany), and the filter resuspended in 10 mL of the same water, then rubbed with a sterile stick and vortexed for 2 min to detach bacteria. Aliquots of 1 mL were treated at 50 ± 1°C for 30 min to inactivate interfering microorganisms; then, volumes of 0.1 mL of the untreated and treated samples were spread on *Legionella* CYE Agar Base (Oxoid, Milan, Italy), added with BCYE growth supplement and GVPC selective supplement (Oxoid). All the plates were incubated at 37°C inside jars under modified atmosphere (2.5% CO_2) for at least 10 days; suspected colonies (CFU) were counted and subjected to preliminary differentiation test. Typical colonies of Legionella appear white-grey colour, small and slightly convex, with a ground-glass appearance. Only colonies of this type were sub-cultured on CYE Agar with and without L-cysteine at 37°C for 48 h. The inability to grow on CYE agar base without BCYE growth supplement confirmed the *Legionella* positivity of the sample.

Serogroup identification

The colonies identified as presumptive *Legionella* were confirmed at the species and serogroup level with the agglutination latex test (*Legionella* Latex Test Kit, Oxoid), generally used for the identification of predominant *Legionella* species from patients with suspected legionellosis or environmental sources. The test allows the separate identification of *L. pneumophila* serogroup 1, *L. pneumophila* serogroups 2–14 and of seven other *Legionella* (polyvalent) species implicated in human disease (17). Each Kit contains positive and negative control for *Legionella*, which were used each time the Kit was employed.

Statistical Analysis

The analyses were performed by GraphPad Prism 5.0 (GraphPad Inc., La Jolla, CA, USA). Comparison between groups was made using Student's t-test to compare paired data, with Bonferroni's correction for

multiple comparisons. P values <0.05 were considered to be statistically significant.

Results

In the present work, 298 water samples collected from different types of non-hospital facilities were examined. As regards the chemical-physical characteristic of the collected water samples, it can be reported that the temperature for hot water samples ranged from 42°C to 66°C (mean 49.1 ± 5.1°C), while that of cold water ranged from 12°C to 32°C (mean: 20.3 ± 5.3°C). The mean pH was 7.5 ± 0.47 and the residual chlorine 0.14 ± 0.09. Among the examined samples, 17.8% resulted positive for the presence of *Legionella* spp., mostly collected from retirement homes (28.6%), followed by residential buildings (21.3%) and private and public offices (15.3%). The public fountains resulted always negative for the presence of *Legionella* spp. (Table 1). According to the Italian Guidelines for prevention and control of legionellosis (14), the sites resulting positive for *Legionella* spp. were subjected to a decontamination procedure by the hyperchlorination technique and, as expected, in the following microbiological analysis all the water samples resulted negative (data not shown). With regards to the identified *Legionella* serogroups, our results showed the presence of *L. pneumophila* sg 2-14 in most of the analyzed hot water samples (71.7%), while *L. pneumophila* sg 1 was identified in 28.3 % of the samples. It can be observed that the highest percentage of *L. pneumophila* sg 2-14 (87.5%) was identified from samples collected in residential buildings, while the highest percentage of *L. pneumophila* sg 1 (40%) from public and private offices (Table 1).

The different distribution of *Legionella* spp. in the collected hot and cold water samples is presented in Table 2. As shown, the highest percentage of positive samples was obtained from hot water (14.4%). Interestingly, 58.8% of hot water samples collected from retirements homes resulted positive with 3.36 log CFU/L, data that highlight the risk for the elderly living in these facilities. Also 31.8% of hot water collected from residential buildings were found positive for *Legionella* spp. (3.69 log CFU/L), whilst only 3.3% of cold water samples resulted positive for this microorganism. Moreover, the difference between retirement homes, as well as between residential buildings, and private and public offices in the prevalence of *Legionella* was found to be statistically significant.

In accordance to the Italian Guidelines, the results were expressed in Colony Forming Units per litre (CFU/L) with a detection limit of 100 CFU/L. nd: not determined. Asterisks

Table 1 - Number of the collected hot and cold water samples, total positive samples for *Legionella* spp. and related identified serogroups from the different examined facilities.

	Water samples	Hot water	Cold water	Positive samples N (%)	Serogroups	
		N	N		*L. pneumophila* sg 1 N (%)	*L. pneumophila* sg 2-14 N (%)
Public fountains	18	0	18	0.0	0	0
Residential buildings	75	44	31	16 (21.3)	2 (12.5)	14 (87.5)
Public and private offices	163	86	77	25 (15.3)	10 (40)	15 (60)
Retirement homes	42	17	25	12 (28.6)	3 (25)	9 (75)
Total	298	147	151	53 (17.8)	15 (28.3)	38 (71.1)

Table 2 - Distribution of *Legionella* spp. in hot and cold samples from the different examined facilities and related log CFU/L (mean ± sd).

	Percentage of positive samples		log CFU/L	
Facilities	hot water	cold water	hot water	cold water
Public fountains	nd	0.0	nd	0.0
Residential buildings	31.8	3.2	3.69 ± 1.05	3.60 ± 0.12
Public and private offices	21.8	7.8	2.99 ± 0.20*	0.0
Retirement homes	58.8	8.0	3.36 ± 0.34*	2.31 ± 0.19
Total	14.4	3.3	10.04 ± 1.59*	5.91 ± 0.31

represent values statistically significant between the two considered groups (hot water and cold water) by Student's t-test.

Discussion and conclusion

Water risk control is normally focused on disinfection systems to monitor *Legionella* colonization in hospitals and health facilities' water networks, but recent epidemiological data documented that almost 80% of the Legionnaire's disease cases diagnosed in a year are acquired in different locations (13, 18). In Italy, the number of LD cases is underestimated, because specific diagnostic tests are not performed before an antibiotic therapy is started. For this reason, the extension of the prevention activities and water risk controls to residential buildings and other types of places, such as offices and retirement homes, to assess the contamination level of *Legionella* spp. in the water networks became important. It can be observed that, regarding Italy, most of the available literature was referred to hospitals or healthcare facilities (19, 20), but recently several investigations were devoted to understanding the distribution of *Legionella* spp. in other facilities (7, 11, 21).

As regards the epidemiological data of *Legionella* spp. in our Country, the available literature referred to different Italian cities or regions (7, 21, 22), but no data were published on the situation in the Marche region. In any case, from the analysis of the collected data, we can state that our results on *Legionella* positivity in residential buildings (21.3%) are in agreement with those reported by the study of Borella et al. (23) and, more recently, by that of Baggiani et al. (22), referring percentages of *Legionella* positivity in the water of about 20% in the same types of facilities. Concerning retirement homes and offices, the herein reported positivity for *Legionella* (28.6% and 15.3%, respectively) confirmed the distribution of *Legionella* spp. in these water systems of our Country as previously described by other authors. Indeed, De Filippis et al. (7) found *Legionella* spp. in 30.7% of samples from retirement homes in Rome and, similarly, Napoli et al. (21) referred 28.9 % of *Legionella* positivity in water samples from offices in Southern Italy. A comparison of the *L. pneumophila* serotypes herein identified (71.1% *L. pneumophila* sg 2-14 and 28.3% *L. pneumophila* sg 1) with other studies of our region is not possible, but in general, the serogroups recovered in the examined non-hospital facilities are similar to those reported by researchers in different areas of the country (7, 21, 22, 24).

Our data evidenced that the low temperatures do not represent an ideal condition for the growth of *Legionella*, as the highest percentage of positive samples was obtained from hot water (Table 2). Indeed, De Filippis et al. (24) have found *Legionella* spp. only in 2 of the 49 samples (4.0%) with a temperature <30°C. Based on the presented

data, much attention must be directed to the control of the biological risk regarding the presence of *Legionella* spp. in different facilities, such as residential buildings, public and private offices and retirement homes, also considering that infections caused by *L. pneumophila* are acquired in non-hospital community environments (11).

In conclusion, this work is the first one to describe the distribution of *Legionella* spp. in non-hospital facilities (such as residential buildings, public and private offices and retirement homes) of the urban area of Pesaro-Urbino. Overall, the obtained results on the water quality proved the need of a regular and constant control to prevent the microbiological risk for legionellosis, particularly in the facilities housing the elderly, that are considered more susceptible to this infection. A larger study, considering a longer surveillance period, will be necessary to realize a better epidemiological map related to *Legionella* distribution in specific structures.

Authors' contributions. LS, MS and RC are responsible for the design of the study. LS performed the microbiological analysis, MS analyzed the data and RC wrote the paper. All authors reviewed and approved the final version of the manuscript.

Conflict of interest. The authors declare that they have no conflict of interest.

Riassunto

***Isolamento ed identificazione di** Legionella spp**. da strutture non ospedaliere: studio preliminare di sorveglianza annuale nell'area urbana di Pesaro-Urbino (Centro Italia)*

***Premessa.** Legionella* spp è considerata uno dei principali microrganismi responsabili di morbosità e mortalità potenzialmente prevedibili. Questi microrganismi sono ubiquitari, tuttavia sono disponibili solo informazioni incomplete sulla distribuzione geografica delle specie di *Legionella* nella nostra regione.

Disegno dello studio. Per questo motivo, nel presente studio è stata esaminata la distribuzione di *Legionella* spp. in strutture non ospedaliere dell'area urbana di Pesaro-Urbino (Italia Centrale).

Metodi. Sono stati sottoposti a protocolli standard di isolamento ed identificazione un totale di 298 campioni di acqua raccolti da fontane pubbliche, edifici residenziali, uffici pubblici e privati e case di riposo.

Risultati. Dai dati si evince che il 17,8% dei campioni di acqua raccolti risultava positivo per *Legionella* spp. (28,6% provenienti da case di riposo, 21,3% da edifici residenziali, 15.3% da uffici privati e pubblici). La percentuale più alta di campioni positivi (14.4%) è stata trovata in campioni di acqua calda provenienti da case di riposo (58,8%) ed edifici residenziali (31,8%), mentre i sierogruppi più frequentemente isolati sono stati *L. pneumophila* 2-14 (71,7%).

Conclusioni. Questo lavoro è il primo che descrive la distribuzione di *Legionella* spp. nelle strutture non ospedaliere nella provincia di Pesaro-Urbino ed evidenzia una condizione di rischio potenziale per le categorie fragili, indicando quindi la necessità di un controllo regolare e costante per prevenire il rischio microbiologico di legionellosi, in particolare nelle strutture che ospitano stabilmente persone anziane.

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